Characterization of Gene *rsp_0003*'s Impact on *Rhodobacter sphaeroides'* Growth with D-lactate

Research Thesis

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by

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Abstract

This study was conducted using *Rhodobacter sphaeroides*, due to its ability to utilize a variety of carbon substrates which are converted to different central carbon intermediates. Among the different carbon substrates are D-lactate and L-lactate, which are proposed to both be converted to pyruvate in central carbon metabolism. Despite this, a transposon mutant (Au18KH24) was isolated that behaved differently when grown with D-lactate and L-lactate. The isolated transposon mutant grew aerobically and phototrophically (or anaerobically in the light) with L-lactate, but had compromised aerobic and phototrophic growth with D-lactate. Interestingly, growth with D-lactate was recovered after a prolonged incubation. The phenotypic suppression may be due to genetic factors or the adaptation of the transposon mutant to the environment within the liquid culture. The transposon insertion for Au18KH24 was mapped to *rsp_0003*. RSP_0003 is a multi-domain protein, whose initial 150 residues are annotated as belonging to the "Serine Recombinase" family (E-value: 4.59e-19; Accession ID: cd00338). Based on nucleotide spacing between the coding regions, *rsp_0003* is likely co-transcribed, along with *rsp_0002* and *rsp_0004*. The location of *rsp_0003* suggests that a transposon insertion could impact the other genes located within the gene cluster. The possible polarizing effects on *rsp_0002* and *rsp_0004* should be taken into consideration, given that the mutant Au18KH24 has yet to be complemented. The overall purpose of this study is to characterize *rsp_0003*, as well as understand *rsp_0003*'s role for growth with D-lactate. This will allow us to further understand the differences between D-lactate and L-lactate metabolism.

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Introduction

Rhodobacter sphaeroides is a phototrophic purple non-sulfur bacterium with the unique capability of using different carbon compounds as the sole carbon substrate for growth[1]. Not only is *R. sphaeroides* capable of using these different carbon substrates as a source of carbon during phototrophic growth, but it also uses different carbon substrates, in addition, as a source of energy during respiratory growth. With the change of external growth conditions, that is, growth anaerobically in the light, *R. sphaeroides* can use all available carbon for cellular components. The ability to make this distinction between energy usage and carbon assimilation makes *R. sphaeroides* an ideal organism for studying carbon metabolism.

Among the many carbon substrates that *R. sphaeroides* can utilize are L-lactate and D-lactate. Both of these carbon substrates are converted to be usable in central carbon metabolism. The metabolic pathways leading into central carbon metabolism though can be different, despite these two compounds only differing in the spatial arrangement of their atoms. In this study, we discuss a mutant of *R. sphaeroides* with an interruption in *rsp_0003*. This mutant demonstrated the ability to utilize L-lactate as a source of carbon, but had impaired growth with D-lactate. Interestingly, the mutant's growth with D-lactate was restored following a long lag phase. The attributes of this mutant of *R. sphaeroides*, ultimately, led us to investigate the role of the gene product of *rsp_0003*.

In the process of determining the function of *rsp_0003*'s gene product, the role of metabolic regulation was taken into consideration. There are a variety of ways that metabolism can be regulated, including global or local regulators that can modulate which genes are expressed in order to manipulate metabolic pathways as a response to growth conditions[2]. These regulators can be transcription factors that regulate the transcription of genes by binding DNA.

The activity of the transcriptional regulator can be modulated by binding of an effector molecule or by covalent modification. In turn the transcription factor operates like a switch to either increase or decrease expression of a gene target. Additionally, the possibility of allosteric regulation of a D-lactate oxidizing enzyme or the activity of a D-lactate-specific uptake system was taken into consideration. Allosteric regulation would involve the binding of an effector molecule to the protein product, not at the active site, in order to modulate the activity of the D-lactate specific protein. Within the natural environment of *R. sphaeroides*, a mixture of carbon substrates is usually available. This may lend itself to *R. sphaeroides* modulating which carbon substrates are utilized at any given time.

The possible functions of RSP_0003 serve as a framework for understanding the dynamic potential of *rsp_0003* regarding *R. sphaeroides*' metabolism. The aim of this study overall is to analyze and characterize a mutant of *R. sphaeroides* that possesses an interruption in *rsp_0003* in order to gain a greater understanding of the role that its gene product may play in the metabolism of D-lactate. Furthermore, this will be beneficial in demarcating the differences between the metabolic pathways involving D-lactate and L-lactate.

Materials and Methods

Materials. All bacterial strains, plasmid vectors, and primers used are listed in Table S2, S3, and S4, respectively.

Bacterial strains and growth conditions. *Rhodobacter sphaeroides* 2.4.1 (DSMZ 158) was grown anaerobically in the light (3,000 lx) or aerobically in the dark at pH 6.7 and 30 °C. The oxygen in Hungate tubes was removed and replaced with N_2 in order to make the anaerobic environment. *Escherichia coli* strain cells were grown aerobically at 37 °C. Growth occurred on minimal media supplemented with a 10 mM concentration of the desired carbon source. The sodium salt of the carboxylic acids were used. The minimal media was made for 1 L (the final concentrations are given in parentheses) by adding 1.2 g NH_4Cl (22mM), 0.2 g $MgSO_4 \cdot 7H_2O$ (0.8 mM), 0.07 g $CaCl_2 \cdot 2H_2O$ (0.5 mM), and additional compounds in the concentrations described in Table S1[3]. For *R. sphaeroides*, kanamycin (20 μg/mL) was added to the minimal media as needed. For *E. coli*, kanamycin (50 μg/mL) was added to Luria broth (LB), comprised of 10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl (pH 7.0), as needed.

Random transposon mutagenesis of *Rhodobacter sphaeroides*. *E. coli* BW20767 cells, which contain the pRL27 suicide vector, and wild-type *R. sphaeroides* 2.4.1 were grown using LB media and harvested during exponential growth phase. Equal amounts of cells (based on optical density) were combined in liquid media, concentrated, and then placed as a single drop on a LB plate containing no antibiotic. Following overnight aerobic growth at 30 °C in the dark, the strain mixture was harvested. Serial dilutions were then performed and the dilutions were spread-plated on minimal media supplemented with L-malate-kanamycin and incubated at 30 °C aerobically in the dark for several days. The minimal media L-malate-kanamycin plates produced isolated

colonies of the strain mixture that were screened for growth aerobically via patching on minimal media plates containing kanamycin. The plates were each additionally supplemented with one of these carbon sources: L-malate (control), D-malate, L-lactate, D-lactate, or acetate. Growth behavior was observed with each carbon source. A mutant was chosen for aerobic and phototrophic rescreening when its growth behavior deviated from that of the control plate (L-malate).

Identification of the transposon insertion within the genome. Isolates with phenotypes of interest were chosen to have genomic DNA isolated from them. Genomic DNA was isolated using the GeneJet DNA purification kit. Following isolation of genomic DNA, 34 µL of the samples were digested with the *Nco*I restriction enzyme (addition of 4 µL of 10× *Nco*I buffer and 2 µL *Nco*I, incubation for 6 – 8 hours at 37 °C), the enzyme was then heat-inactivated by incubation for 15 minutes at 80 °C, 10 µL were removed to check for complete digestion via gel electrophoresis, and the remaining mixture was diluted with 148 µL water and the DNA was ligated using T4 DNA ligase (addition of 20 µL of 10× T4 DNA ligase buffer and 2 µL T4 DNA ligase, incubation at room temperature overnight). Chemically-competent *E. coli* DH5αλpir cells were transformed with the ligated products. The transformants were spread plated on LB medium containing kanamycin. The plasmid was isolated using the GeneJet plasmid isolation kit. The size of the plasmid was determined after an *Nco*I test digest and analysis by a 0.8% agarose gel electrophoresis. The concentration of the plasmid DNA was determined using a NanoDrop spectrophotometer, measured in ng/µL. The plasmid containing the transposon insertion was sent for Sanger sequencing (Genewiz), utilizing primer tnpRL_17_1 and primer tnpRL_13_2. The gene interrupted by the transposon insertion was determined via a DNA sequence similarity search on NCBI BLAST.

Transformation protocol. A suitable amount of DNA (1 μL of plasmid or 30 – 50 μL of a ligation mixture) was combined with freshly thawed 200 μL of chemically competent *E. coli* cells. The mixture was placed on ice for 20 minutes, followed by two minutes at 42 °C, and then was placed back on ice for 10 minutes. An aliquot of 800 μL of LB liquid media was added to the chilled cells and incubated for one hour at 37 °C. The cells were subsequently spread plated on LB solid media containing kanamycin (50 μg/mL). The LB plates were incubated overnight at 37 °C to select for desired transformants.

Growth experiments. Cells were pregrown phototrophically (or anaerobically in the light) in 4.5 mL of liquid minimal media containing 10 mM L-malate or L-lactate. The cells were grown until they reached stationary phase (OD_{578} of ~1.3). Once in stationary phase for at least 5 hours, 0.15 mL of cells were then transferred to a new Hungate tube (for phototrophic growth) or test tube with a metal cap (for aerobic growth) containing 4.5 mL of minimal media and a supplementary carbon source. Growth was measured by determining the optical density at 578 nm (OD_{578}), which was taken every few hours, with more readings being taken during the exponential growth phase. Once the cells reached a reading equivalent to stationary phase, a final reading was taken one day after or even longer, depending on the growth experiment. If the OD reading was >1, the OD was determined from a sample of cells that were removed from the Hungate tube or test tube with a metal cap and mixed in a 1:10 dilution.

Additional growth experiments were conducted to determine the precise time at which Au18KH24 (a transposon mutant isolate) had a supposed change in growth behavior from no growth to growth. Growth was measured via optical density as previously described. A Hungate tube containing 4.5 mL of liquid minimal media supplemented with D-lactate was grown phototrophically. At intervals, a sample of 0.2 mL of cells was taken from the Hungate tube and

re-inoculated into a new tube containing the same liquid media. These cells were then grown phototrophically and growth was measured via optical density. When a sample culture was taken that grew quickly after re-inoculation, the sample culture was plated on media containing L-lactate (non-selective conditions). A stock culture was prepared from an isolated colony for each sample culture plated on media containing L-lactate (non-selective conditions). The genomic DNA was subsequently isolated for each sample culture.

Results

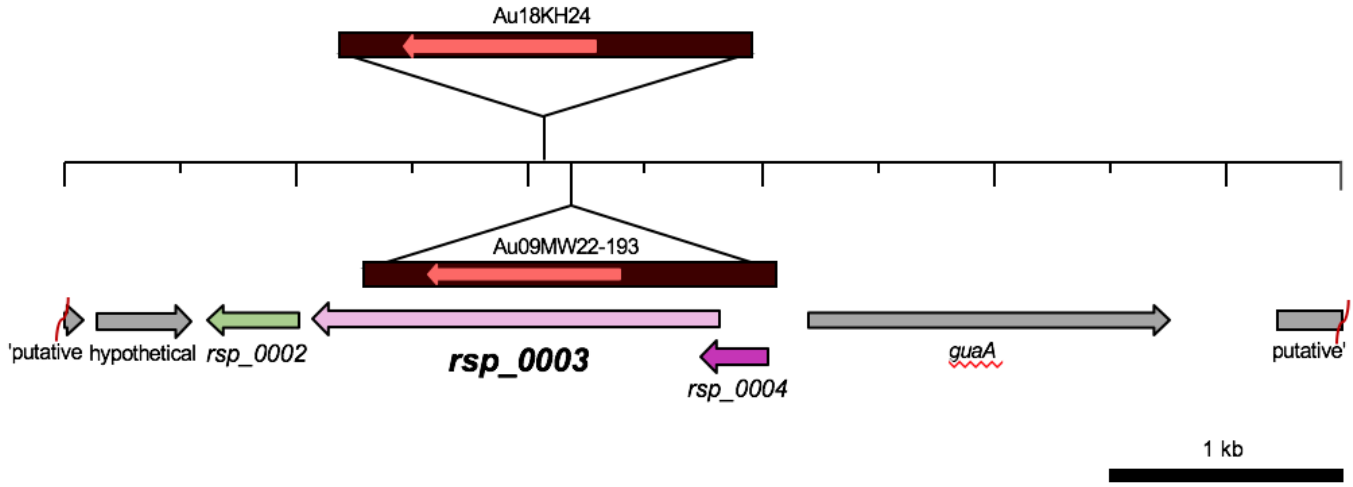

Figure 1. Genomic region of *R. sphaeroides* containing the transposon insertion in *rsp_0003*. Two different mutants were isolated containing an interruption in *rsp_0003*: Au18KH24 and Au09MW22-193. The direction of the kanamycin resistance gene is indicated by the red arrow. The gene *rsp_0003* has a recombinase-like domain associated with the first 450 nucleotides of the coding region. A discrete genomic region, *rsp_0004*, is shown overlapping *rsp_0003*. The genes *rsp_0002*, *rsp_0003*, and *rsp_0004*, are suspected to be co-transcribed. The gene *guaA* is annotated to encode for a "GMP synthase".

Transposon mutagenesis resulting in a transposon mutant library. Transposon mutagenesis of *R. sphaeroides* 2.4.1 was essential for the generation of mutants with observable phenotypes which deviated from that of the wild type. The mini-Tn5 transposon was delivered into *R. sphaeroides* 2.4.1 via conjugation with *E. coli* BW20767, carrying the plasmid pRL27. The pRL27 vector contains a modified T5 transposase gene (*tnp*) that encodes for a transposase that is 1,000-fold more active than the wild-type transposase[4]. The transposase gene is under the control of the *tetA* promoter (*tetA*p), which is functional in a wide range of organisms. The increased activity of the transposase allows for efficient transposon delivery into the *R. sphaeroides* 2.4.1 genome. The transposon itself also contains a gene conferring kanamycin resistance (*aph*), that was utilized as a selectable marker during screening, and the origin of

replication from plasmid R6K (*ori*R6K), which allows for cloning of the transposon insertion mutants. Once transposon mutants of *R. sphaeroides* 2.4.1 were generated, they were screened on minimal media plates containing different carbon substrates: L-malate, D-malate, L-lactate, D-lactate and acetate. Mutant phenotypes observed during the screen could be indicative of changes in metabolic pathways due to genotypic alterations caused by the transposon insertion.

Characterization of the genomic region of *R. sphaeroides* possessing transposon mutants with interruptions in *rsp_0003*.A transposon mutant of *R. sphaeroides* that grew anaerobically on plates in the light with L-lactate but exhibited no growth with D-lactate as the sole carbon source was isolated from a transposon library and was named Au18KH24. An additional transposon mutant, named Au09MW22-193, was selected from the pre-existing transposon library for further characterization. The site of the transposon insertion for both mutants was identified within *rsp_0003* on chromosome 1 of *R. sphaeroides* (accession number NC_007493). The identified gene, *rsp_0003*, encodes for RSP_0003, which is a multi-domain protein (accession number YP_353073). The initial 150 residues of RSP_0003 are annotated as belonging to the "Serine Recombinase" family (E-value: 4.59e-19; Accession ID: cd00338). The gene *rsp_0004* is only 291 bp (base pairs) long and encodes for a hypothetical protein with no known function. The gene *rsp_0002* is annotated to encode for a "histone-like nucleoid-structuring protein" and belong to the "Histone_HNS" family (E-value: 2.18e-25; Accession ID: pfam00816). These genes are important to mention given that they may be co-transcribed with *rsp_0003* and their functions within this cluster are not immediately apparent.

Another region important to note is the genomic region downstream of the suspected gene cluster consisting of *rsp_0002*, *rsp_0003*, and *rsp_0004*. There are 24 possible genes all oriented in the same direction, with minimal spacing between their coding regions, that are

suspected to be co-transcribed. While a majority of the genes within the cluster are annotated to encode for “hypothetical proteins” or “proteins of unknown function”, there exists genes within the cluster that have phage-related functionality. The gene *rsp_2998* is annotated as a “Phage Terminase” and belongs to the “Terminase_1” superfamily (E-value: 9.28e-58; Accession ID: pfam00354). The gene *rsp_6246*, also located within the gene cluster, is annotated as a “phage head-tail joining protein” and is suspected to belong to the “Phage_H_T_join” superfamily (E-value: 3.67e-10; Accession ID: pfam05521). The presence of a terminase and tail protein are indicators of a possible prophage, due to their necessity for integration of phage DNA within a bacterial cell.

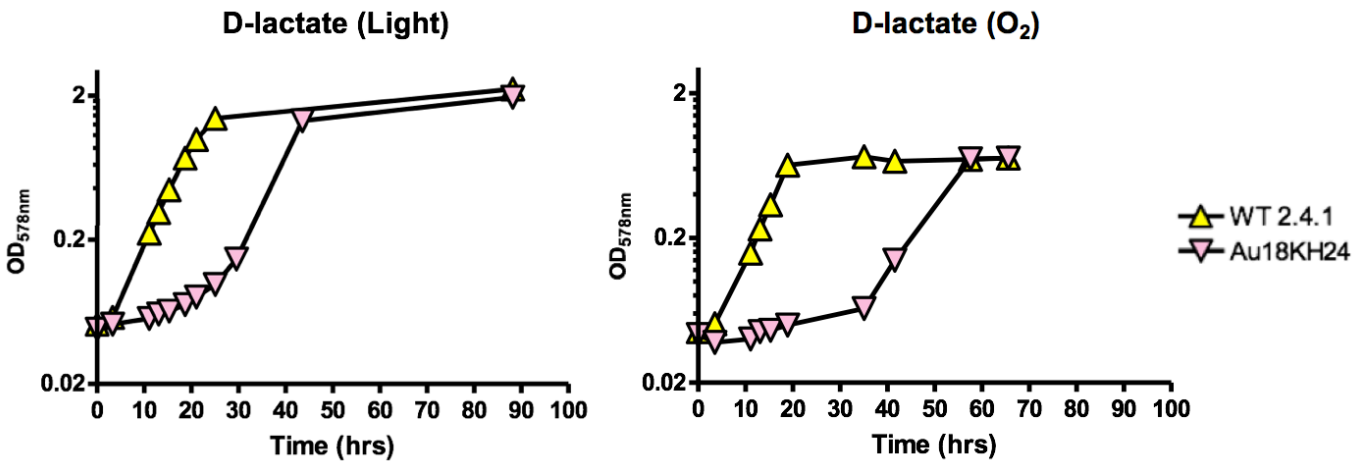

Figure 2. Wild-type *R. sphaeroides* 2.4.1 and mutant Au18KH24 during aerobic (right) and phototrophic (left) liquid culture growth in minimal media supplemented with D-lactate.

Table 1. Doubling time of Au18KH24 versus wild-type *R. sphaeroides* 2.4.1 with D-lactate [a]

	Phototrophic (Light)	Aerobic (O_2)
Wild-type (WT)	4.3 ± 0.2[b]	3.8 ± 0.2[b]
Au18KH24	Cannot be determined	Cannot be determined

[a] Doubling time (h) ± standard deviation is shown for all experiments.
[b] Growth experiment performed only once; not a biological triplicate.

Mutant phenotype of Au18KH24 displaying compromised growth with D-lactate. Aerobic and phototrophic growth of Au18KH24, which carries an interruption in *rsp_0003*, revealed compromised growth in liquid culture minimal media with D-lactate (Figure 2, Table 1). The compromised growth was characterized by a long lag phase (~30 hours). This contrasts the growth of wild-type *R. sphaeroides* 2.4.1, which displayed a short lag phase. When Au18KH24 reached stationary phase, the growth yield appeared similar to that of the wild type. The growth behaviors of the wild-type *R. sphaeroides* 2.4.1 and Au18KH24 differed by the length of their lag phases. The long lag phase of Au18KH24, followed by recovery of growth with D-lactate, may have been due to suppression of the mutant phenotype. The doubling time for Au18KH24 with D-lactate could not be accurately determined due to error exceeding 10 % for calculated doubling times.

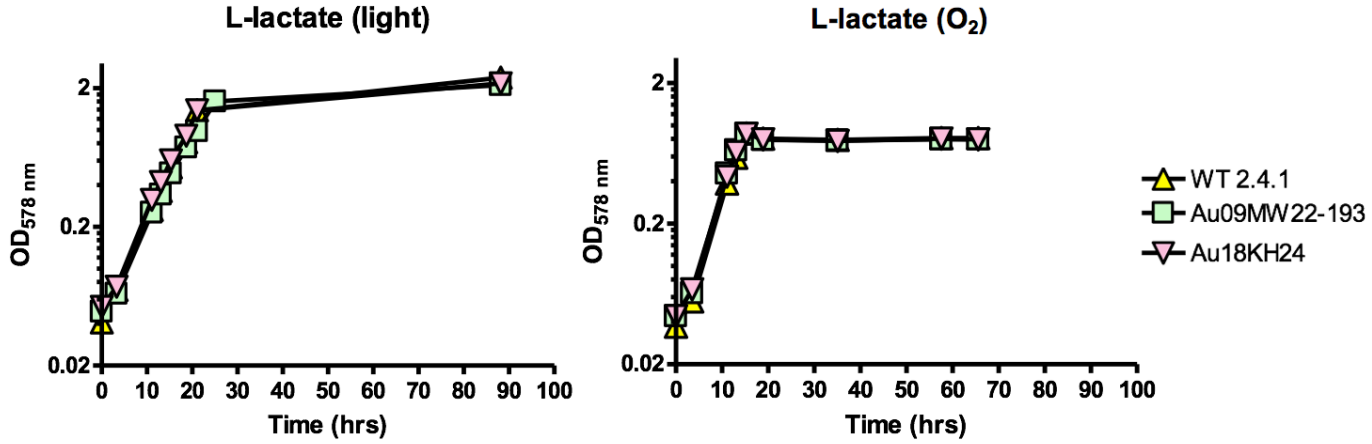

Figure 3. Growth of mutant Au18KH24 aerobically (right) and phototrophically (left) in minimal media liquid culture supplemented with L-lactate. Mutant Au09MW22-193 also has an interruption in *rsp_0003* and exhibits growth behavior similar to wild-type *R. sphaeroides* 2.4.1 and Au18KH24 with L-lactate.

Table 2. Doubling time of Au18KH24 versus wild-type *R. sphaeroides* 2.4.1 with L-lactate [a]

	Phototrophic (Light)	Aerobic (O_2)
Wild-type (WT)	4.2 ± 0.3[b]	3.0 ± 0.3[b]
Au18KH24	4.5 ± 0.3	3.1 ± 0.3
Au09MW22-193	4.6 ± 0.4	3.0 ± 0.5[c]

[a] Doubling time (h) ± standard deviation is shown for all experiments.
[b] Growth experiment performed only once; not a biological triplicate.
[c] Standard deviation exceeds range of 10 % error.

Table 3. Doubling time of Au18KH24 versus wild-type *R. sphaeroides* 2.4.1 with L-malate [a]

	Phototrophic (Light)	Aerobic (O_2)
Wild-type (WT)	3.8 ± 0.3[b]	2.6 ± 0.1[b]
Au18KH24	3.4 ± 0.1[b]	2.9 ± 0.4[b,c]

[a] Doubling time (h) ± standard deviation is shown for all experiments.
[b] Growth experiment performed only once; not a biological triplicate.
[c] Standard deviation exceeds range of 10 % error.

Growth with L-lactate shows no apparent impairment due to interruption of *rsp_0003*.

D-lactate and L-lactate are stereoisomers that differ only in the spatial arrangement of their atoms. Despite this, mutant Au18KH24 did not have impaired growth with L-lactate (Figure 3, Table 2). Au18KH24 exhibited growth with L-lactate that is similar to wild type. Additionally, Au18KH24 was tested on other growth substrates, such as L-malate, and also exhibited growth

similar to wild type (Table 3). This suggests that the growth defect for Au18KH24 is D-lactate specific.

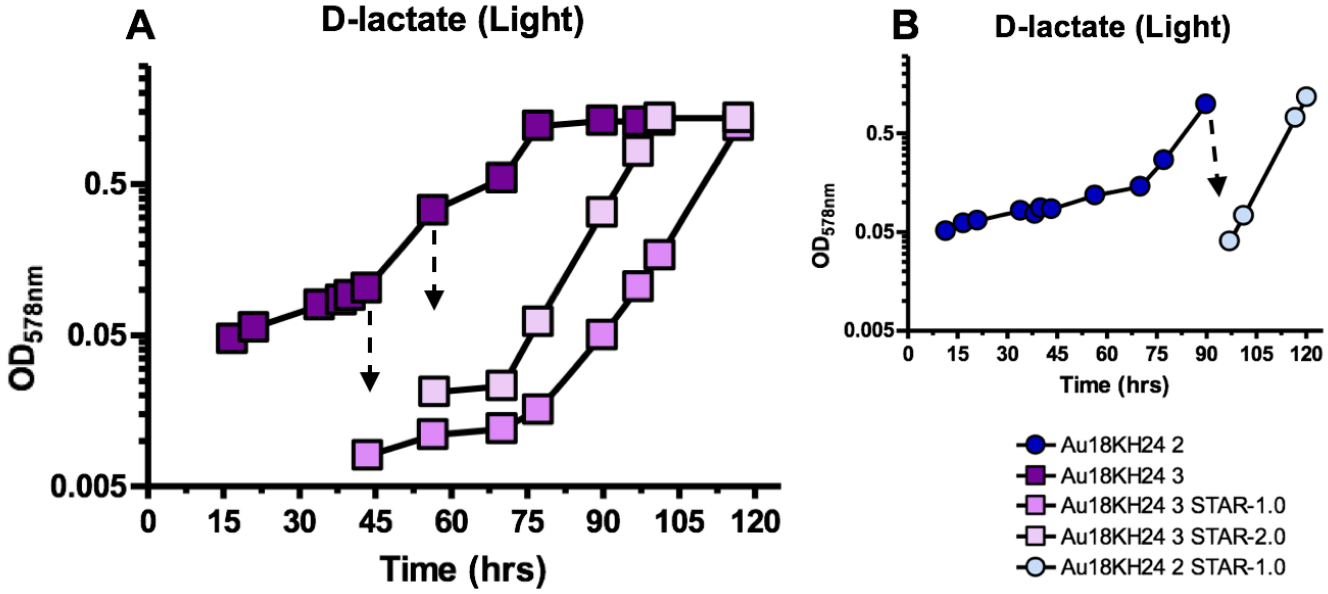

Figure 4. Photoheterotrophic growth of minimal media liquid cultures of mutant Au18KH24, with D-lactate as the sole carbon source. Black arrows indicate the first reading for re-inoculated cultures. (A) An initial culture (dark purple square) of mutant Au18KH24 was re-inoculated at two separate time points: Au18KH24 3 STAR 1.0 (medium purple square) and Au18KH24 3 STAR 2.0 (light purple square). (B) In a separate experiment, Mutant Au18KH24 was re-inoculated (light blue circle) after the initial culture (dark blue circle) had been grown late into the exponential growth phase.

Restoration of wild-type growth for Au18KH24 with D-lactate. In order to test for possible phenotypic suppression of Au18KH24's mutant phenotype, the photoheterotrophic growth of liquid culture minimal media with D-lactate was measured. The test showed that liquid culture samples taken at different time points during the exponential growth phase had different length lag phases following re-inoculation. The liquid culture samples that were taken earlier in the exponential growth phase displayed an apparent lag phase following re-inoculation (Figure 4). The liquid culture samples taken near the end of the exponential growth phase had rescued growth with D-lactate immediately following re-inoculation. All liquid culture samples eventually recovered growth with D-lactate though, which is consistent with a possible genetic suppressor mutation resulting in the phenotypic suppression observed.

The cells carrying the suppressor mutation would need to multiply over several generations in order to have a measurable change in OD. This would explain why the liquid culture samples taken early on in the exponential growth phase had a longer lag phase, but still recovered growth with D-lactate over time. Those liquid culture samples taken near the end of the exponential growth phase would have already had a large population of cells with the suppressor mutation, resulting in immediate growth with D-lactate upon re-inoculation. This growth behavior indicates a potential genetic suppression that allows for Au18KH24 to have restored growth with D-lactate.

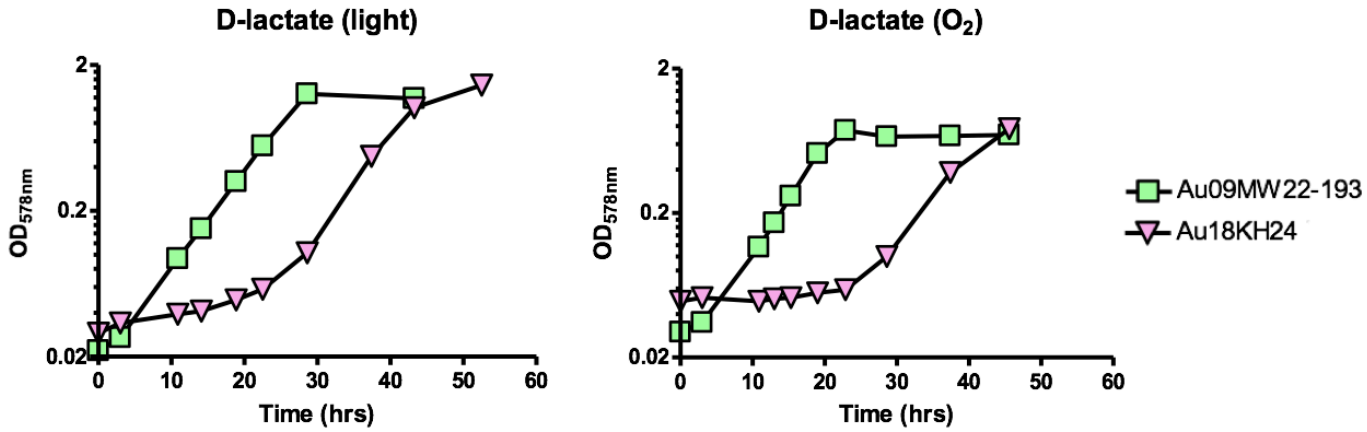

Figure 5. Mutant Au18KH24's compromised growth aerobically (right) and phototrophically (left) in liquid culture minimal media with D-lactate. Mutant Au09MW22-193 exhibited growth similar to wild-type *R. sphaeroides* 2.4.1 with D-lactate.

Table 4. Doubling time of Au09MW22-193 versus wild-type *R. sphaeroides* 2.4.1 with D-lactate [a]

	Phototrophic (Light)	Aerobic (O_2)
Wild-type (WT)	4.3 ± 0.2[b]	3.8 ± 0.2[b]
Au09MW22-193	4.5 ± 0.1[b]	3.8 ± 0.1[b]

[a] Doubling time (h) ± standard deviation is shown for all experiments.
[b] Growth experiment performed only once; not a biological triplicate.

Mutant Au09MW22-193 has an interruption in *rsp_0003* but does not have compromised growth with D-lactate. Both mutant Au18KH24 and Au09MW22-193 had interruptions in *rsp_0003* (Figure 1). Mutant Au18KH24 though had compromised growth with D-lactate, whereas Au09MW22-193 did not exhibit compromised growth (Figure 5, Table 4). It should be noted that both Au18KH24 and Au09MW22-193 had wild-type-like growth with L-lactate (Figure 3, Table 2). This data is surprising considering both mutants have an interruption in the same gene.

Discussion

Two mutants with a transposon insertion in *rsp_0003* differed in their phenotypes. In this study, the transposon mutant Au18KH24 was isolated that initially showed no growth with D-lactate but exhibited wild-type growth with L-lactate. Further characterization of this mutant revealed that the D-lactate-negative phenotype of Au18KH24 was suppressed after prolonged incubation either aerobically in the dark or anaerobically in the light, when grown in liquid media supplemented with D-lactate as the sole carbon source. The insertion site of the transposon for Au18KH24 was mapped to the coding region of *rsp_0003* (Figure 1). As part of a teaching class, another transposon mutant, Au09MW22-193, was recovered with the same *rsp_0003* gene interrupted. That is why it came as a surprise when Au18KH24 and Au09MW22-193, which both had interruptions in *rsp_0003*, behaved differently when grown with D-lactate (Figure 5). While growth was impaired for Au18KH24, the growth of Au09MW22-193 still mirrored that of the wild type.

The insertion of the transposon for Au09MW22-193 was upstream of the Au18KH24 transposon insertion site (Figure 1). Due to the insertion site of Au09MW22-193 being positioned upstream of Au18KH24, the gene product of *rsp_0003* for the Au09MW22-193 mutant is expected to be shorter, in comparison to the gene product of the Au18KH24 mutant. Both mutants would hypothetically still have *rsp_0004* intact as it is earlier on in the gene cluster than *rsp_0003*. The insertion of the transposon for both mutants would also likely have polarizing effects on *rsp_0002*, given that the genes are likely co-transcribed.

There are a few possible explanations for the different growth behaviors of Au18KH24 and Au09MW22-193. The first being that Au09MW22-193 has already had a spontaneous second site mutation that suppresses the no growth phenotype with D-lactate. An alternative

explanation though could be that Au18KH24 contains another transposon insertion that is responsible for the D-lactate-negative phenotype.

A Southern blot could be conducted to determine if there is a second transposon insertion site. The Au18KH24 mutant's genome would be fragmented using *Nco*I and the results would be visualized as bands of fragmented DNA via gel electrophoresis. The DNA from the gel would be transferred onto a nitrocellulose membrane and denatured, in order for the single-stranded radioactively-labeled probe to hybridize. The *Nco*I fragment(s) carrying the transposon would be identified by hybridization of a single-stranded radioactively-labeled probe containing the transposon DNA. If the radioactive-labeled probe containing the transposon is hybridized to more than one place on the genome, when visualized on X-ray film, then there was likely more than one transposon insertion in Au18KH24. A transposon insertion would appear as a band at approximately 7000 bp that fluoresces on X-ray film.

An attempted complementation of the mutant Au18KH24 could be performed by introducing a plasmid containing the coding regions for *rsp_0004*, *rsp_0003*, and *rsp_0002*, together, along with approximately 200 nucleotides upstream of the gene cluster, which should serve as a promoter. The presence of growth with D-lactate without a lag phase would indicate the transposon insertion within *rsp_0003* was responsible for the D-lactate-compromised growth. Additionally, a clean deletion of *rsp_0003*, or the entire gene cluster, may be constructed to determine if the Au18KH24 mutation is reciprocated.

An unlikely hypothesis, but important to include nonetheless, explaining the differences in phenotype of the two mutants is that *rsp_0003* has a genotypic link to *rsp_0004*. The "hypothetical protein" product of rsp_*0004*'s gene could be a short regulatory sequence that regulates the rest of the genes in the cluster. When the transposon was inserted into *rsp_0003* for

Au18KH24, an essential sequence for recognition by RSP_0004 may have been interrupted as well. This resulted in the inability of RSP_0004 to act on *rsp_0003*. However, that sequence would have remained intact for Au09MW22-193, due to the transposon insertion being upstream of the transposon insertion of Au18KH24. This would result in RSP_0004 still being able to recognize the essential sequence. RSP_0004 could then exercise its regulatory function on *rsp_0003*. Further characterization of the function of RSP_0004 would need to be conducted in order to determine if this is a possible reason for the observed phenotypes of both mutants.

The previously stated hypothesizes explaining the differences between growth behavior with D-lactate for mutant Au18KH24 and Au09MW22-193 are important to keep in mind. Moving forward though, through the rest of the discussion, we will assume that the interruption of the *rsp_0003* gene is responsible for the observed D-lactate-negative phenotype before apparent suppression.

RSP_0003 contributes to growth with D-lactate. Studies involving *Pseudomonas putida* have shown that D-lactate can be oxidized to pyruvate via a bacterial membrane-associated NAD-independent D-lactate dehydrogenase[5]. Other organisms have been shown to have lactate oxidizing enzymes that have specificity for D-lactate or L-lactate. Additional studies involving *Escherichia coli* have shown that enzymes, such as glycolate oxidoreductase, preferentially oxidize D-lactate over L-lactate[6]. The enzymatic rate of activity was high when utilizing D-lactate as the carbon substrate and low when utilizing L-lactate as the substrate. This is an indication of the differences in metabolic pathways and, by extension, the catalytic enzymes used in those pathways to convert D-lactate and L-lactate, respectively. This specificity is important for the examination of the functional role of RSP_0003.

Analysis of growth data suggests that the gene product of *rsp_0003* plays a role in growth with D-lactate, both aerobically and phototrophically. Au18KH24 had a long lag time of approximately 30 hours before the exponential growth phase began, which contrasted the wild-type *R. sphaeroides* 2.4.1 that grew almost immediately following inoculation. The eventual recovery of growth for mutant Au18KH24 though shows that the interruption of *rsp_0003* results in slow or impaired growth, but not no-growth, with D-lactate.

It could be expected that a mutation in a gene that encodes for the only D-lactate oxidizing enzyme for a strain of bacterium would result in a mutant that could no longer grow with D-lactate. There is evidence that a recently characterized gene, *rsp_1018*, encodes for a subunit of a multimeric D-lactate oxidizing enzyme within *R. sphaeroides*[7]. When *rsp_1018* was interrupted, the resulting mutant displayed a no-growth phenotype with D-lactate. The resulting mutant was subsequently complemented with a functional copy of *rsp_1018*, which restored growth with D-lactate. Since *rsp_0003* does not display the growth behavior of *rsp_1018*, it is highly unlikely that *rsp_0003* encodes for another D-lactate oxidizing enzyme operating within *R. sphaeroides*. As additional evidence, none of the gene products of genes within the suspected gene cluster (*rsp_0004*, *rsp_0003*, and *rsp_0002*) appear to represent redox-active enzyme. Even without direct impact on the oxidation of D-lactate, it is still clear that the gene product, RSP_0003, has some capacity to influence growth with D-lactate for *R. sphaeroides*.

Interruption of *rsp_0003* does not impact growth with L-lactate. As previously stated, D-lactate and L-lactate are enantiomers. This may lead to the belief that these two compounds utilize the same enzymes within their metabolic pathways. However, as demonstrated by Au18KH24's growth with L-lactate, this is likely not the case. Au18KH24 was grown both aerobically and phototrophically with L-lactate. Growth with both conditions mirrored that of

wild-type *R. sphaeroides* 2.4.1 in terms of growth yield and doubling time (Figure 3, Table 2). This suggests that *rsp_0003*'s gene product has a function that impacts only growth with D-lactate, not L-lactate. This provides an example of the many potential differences between D-lactate and L-lactate metabolism.

RSP_0002, RSP_0003, and RSP_0004 are not likely to be transport proteins. Earlier in the discussion, studies were mentioned that indicated differences between the metabolic pathways of D-lactate and L-lactate. It would not be unreasonable to suggest that these differences may also extend to the way these stereoisomeric substrates are taken up into the cell. Transport proteins have an essential role in assisting with the movement of compounds across membranes. These transport systems have protein components with integral transmembrane regions that allow them to facilitate movement across the membrane. The gene *rsp_0003* and *rsp_0002* are not likely to encode for one of these transport proteins given computation analysis of the amino acid sequence of RSP_0003 and RSP_0002 that revealed no transmembrane regions. When the amino acid sequence for the gene product of *rsp_0004*, RSP_0004, was analyzed there was only a 13.9 % chance that less than 10 residues of the protein had a transmembrane region. Therefore, while there is a slight change that RSP_0004 has any residues associated with a transmembrane region, it is highly unlikely.

The capacity of RSP_0003 and RSP_0002 to perform regulatory functions. The *rsp_0003* gene is annotated as a "site-specific recombinase" which would require a portion of the gene product to have a DNA-binding domain in order to function. The gene suspected to be co-transcribed with *rsp_0003* is *rsp_0002*. The gene product of *rsp_0002*, RSP_0002, is annotated as a "histone-like nucleoid-structuring protein". RSP_0002 would also have to have a DNA-binding domain in order to function appropriately. The presence of DNA-binding domains

within both RSP_0003 and RSP_0002 allows for the possibility of regulatory function. However, a gene product's ability to regulate locally or globally cannot be delineated by amino acid sequence alone, therefore, further analysis would be necessary to explore the role of *rsp_0003* and *rsp_0002* as possible regulators.

Rescued growth with D-lactate may be caused by intragenic or extragenic suppression of *rsp_0003*. Liquid culture minimal media supplemented with D-lactate and inoculated with Au18KH24 had samples taken at different time points (Figure 4). Those liquid culture samples taken exhibited different growth behaviors when re-inoculated depending on when the sample was taken. Samples taken earlier in the exponential growth phase lagged for longer before entering the exponential growth phase. In contrast, samples taken late in exponential growth phase, when more cells would have entered stationary phase, such as in Figure 4-B, grew quickly after re-inoculation.

This sudden recovery of growth with a sole carbon substrate has been shown with *Escherichia coli*. In studies using lactose, the wild-type phenotype of growth for *E. coli* has been recovered following a suppressor mutation[8]. The growth behavior of mutant Au18KH24 could have also been caused by an intragenic or extragenic suppressor mutation that occurred early on during the exponential growth phase, but was not observable until cells carrying the suppressor mutation had multiplied over several generations. This second site mutation would ultimately cause the mutant phenotype to revert to the wild-type phenotype of *R. sphaeroides*.

The presence of more cells in stationary phase, of the liquid culture used for re-inoculation, is important to mention given that there is a subset of genetic mutations called adaptive (stationary phase) mutations which allow slowly growing or non-growing cultures the capability to recover growth with the same carbon substrate that they previously could not grow

on[8]. Adaptive mutations have been shown to form mostly via mechanisms similar to mutations in growing (exponential growth phase) cells, with the exception of three experimental systems within *E. coli*. Those experimental systems involved transposon-mediated deletions, substitution mutations in old colonies, and a *lac* frameshift reversion[9,10]. The unique system with *lac* in particular required intervention by recombination proteins and DNA polymerase IV in order to proceed. The only one of these experimental systems that would be potentially applicable to Au18KH24 is the possibility of substitution mutations in old colonies. However, within that study utilizing *E. coli*, the cells were intentionally exposed to DNA-damaging agents to induce an SOS response[10], which did not occur within our study. For these reasons, the mechanisms by which an adaptive mutation would have reverted the D-lactate-negative mutant phenotype of Au18KH24 to the D-lactate-positive wild-type phenotype, will be treated the same as an intragenic or extragenic suppression.

Au18KH24 that grew when re-inoculated was isolated on non-selective conditions in the presence of L-lactate versus D-lactate, and its genomic DNA will be sequenced. The sequences of these isolated samples need to be aligned with the wild-type sequence of *R. sphaeroides* 2.4.1. Several more of these independently isolated mutants would need to be isolated, from different growth experiments, and aligned with not only with wild-type *R. sphaeroides* 2.4.1, but also with each other. All of the mutants would be examined for common mutations, as well as mutations that would impact the same gene, including the regulatory regions. If a common mutation was found between all of the isolated mutants, an attempted reversion of that mutation would be conducted. The reappearance of the original mutant phenotype could show the possible existence of a suppressor mutation within *R. sphaeroides* that impacts growth with D-lactate.

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Appendix

Table S1. Additive components and concentrations for minimal media[3]

Solution	Composition for 1 Liter	Volume (mL/L)	Concentration in Medium
1 M Phosphate Buffer pH 6.7	1 M K_2HPO_4 1 M KH_2PO_4	15	15 mM
100 x Trace Element Solution	500 mg Disodium EDTA 300 mg $FeSO_4 \cdot 7H_2O$ 3 mg $MnCl_2 \cdot 4H_2O$ 50 mg $CoCl_2 \cdot 6H_2O$ 1 mg $CuCl_2 \cdot 2H_2O$ 2 mg $NiCl_2 \cdot 6H_2O$ 3 mg $Na_2MoO_4 \cdot 2H_2O$ 5 mg $ZnSO_4 \cdot 7H_2O$ 2 mg H_3BO_3 (boric acid)	10	1% each
667 x Vitamin Solution	100 mg Cyanocobalamin (Vitamin B_{12}) 300 mg Pyridoxamine-2 HCl (Vitamin B_6) 100 mg Calcium-D(+)-Pantothenate 200 mg Thiamine chloride (Vitamin B_1) 200 mg Nicotinic acid 80 mg 4-Aminobenzoic acid (Vitamin H_1) 20 mg D(+)-Biotin	1.5	0.15 % each

Table S2. Bacterial strains used in this study[4,11]

Name	Relevance	Reference
Escherichia coli BW20767	Donor strain carrying pRL27	Larsen *et al*. (2002)
Escherichia coli DH5α	Cloning strain	Simon *et al*. (1983)
Escherichia coli DH5αλpir	Cloning strain	Alber Lab
Rhodobacter sphaeroides 2.4.1	Wild type	DSMZ 158
Au18KH24	Transposon mutant	This study
Au09MW22-193	Transposon mutant	Micro 581

Table S3. Plasmids used in this study[12]

Name	Relevance	Reference
pRL27	Suicide plasmid vector	Metcalf *et al*. (1996)

Table S4. Primers used in this study[9]

Primer	DNA Sequence (5' → 3')	Reference
tnpRL17_1	AACAAGCCAGGGATGTAACG	Larsen *et al*. (2002)
tnpRL13_2	CAGCAACACCTTCTTCACGA	Larsen *et al*. (2002)